Antisense Agents

DOI: 10.1002/anie.200602346

An Anticoagulant with Light-Triggered Antidote Activity**

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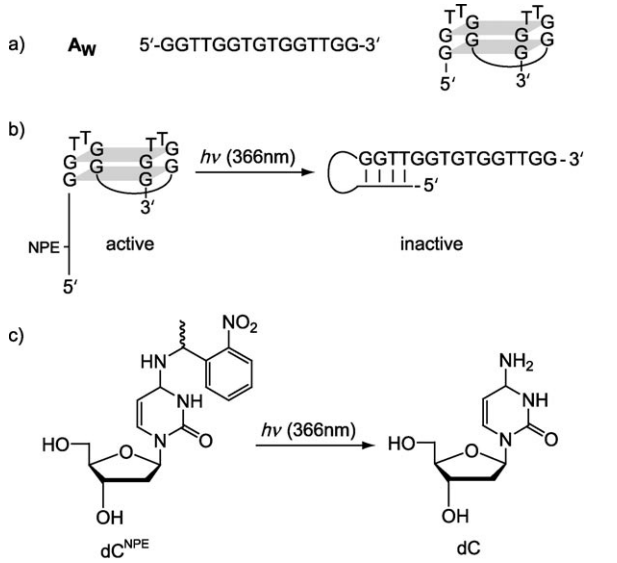
Drug-inhibiting antidotes are helpful tools for the prevention and treatment of unwanted side effects caused by overdosing. These are of particular importance in the case of anticoagulants, the overdosing of which may induce life-threatening bleeding. Among the available anticoagulants, unfractionated heparin is the only one that can be effectively neutralized by a specific antidote. The lack of a specific antidote is a serious disadvantage of all novel anticoagulants (e.g. synthetic heparin, factor Xa, and thrombin inhibitors) even though they are more effective antithrombotics and possess several other advantages over unfractionated heparin.[1] As a consequence unfractionated heparin remains the anticoagulant of choice for patients with a high risk of bleeding or in clinical situations in which rapid reversal of the anticoagulant activity is required, although its use is associated with potential drawbacks including immune reactions.

An alternative approach to the development of antidotes for anticoagulants was introduced recently by Sullenger and co-workers.[2] They used aptamers that target factor IXa of the blood-clotting cascade as effective anticoagulant. Aptamers are short, single-stranded nucleic acids that fold into well-defined three-dimensional shapes.[3] They bind with high affinity and specificity to their respective target molecules and can be used, for example, as potent inhibitors. For the development of an antidote, Sullenger took advantage of the inherent properties of nucleic acids and developed an antisense-based antidote molecule that is able to anneal to the aptamer and thus reverse its inhibitory function.

Another aptamer with anticoagulant activity is the 15mer single-stranded DNA molecule $\mathbf{A_W}$ (Scheme 1a) that binds to and inactivates thrombin, which is a key protein in the blood-clotting cascade.[4] The antithrombin aptamer folds under physiological conditions into a stable G-quadruplex structure and consists of six thymidine and nine guanosine nucleotides. In our studies on the development of methods to control biological processes spatiotemporally with light, we have shown that aptamer activity can be triggered by light after the active site has been temporarily blocked with a photolabile group[5] or the formation of the active conformation of the aptamer has been prevented by modification of a key residue.[6]



Scheme 1. a) The aptamer $\mathbf{A_W}$ binds thrombin selectively and can act as anticoagulant. b) Our concept of an aptamer that contains its own antidote (in an inactive form). The antidote activity is triggered by light irradiation. c) The "caged" nucleic acid residue dC^{NPE}, which acts as a temporary mismatch, synthesized for this purpose. NPE = 1-(2-nitrophenyl)ethyl.

Herein we describe the development of a single oligonucleotide that contains both an aptamer region and a temporarily inactivated "caged" antisense region in the one molecule and can hence act as both an anticoagulant and its own antidote, the latter function being triggered by light. We assumed that variants of the aptamer that bear an elongated 5′- or 3′-region that is complementary to the G-quadruplex sequence are inactive because they are locked in a hairpin conformation rather than folded into the active G-quadruplex (Scheme 1b). However, if the antisense region contains "temporary mismatches",[7] the hairpin is not formed until the molecule is irradiated and the photolabile group is removed.

To ascertain the ideal length of the antisense region we synthesized the aptamer variants $\mathbf{A_1}$–$\mathbf{A_3}$ (Figure 1a). The respective antisense regions are linked to the 5′-end of $\mathbf{A_W}$ by

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[**] This work was made possible by grants of the Fonds der Chemischen Industrie (for A.H. and G.M.) and by an Emmy Noether Fellowship (DFG) for A.H. The authors thank Prof. M. Famulok for his continuing and encouraging support.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

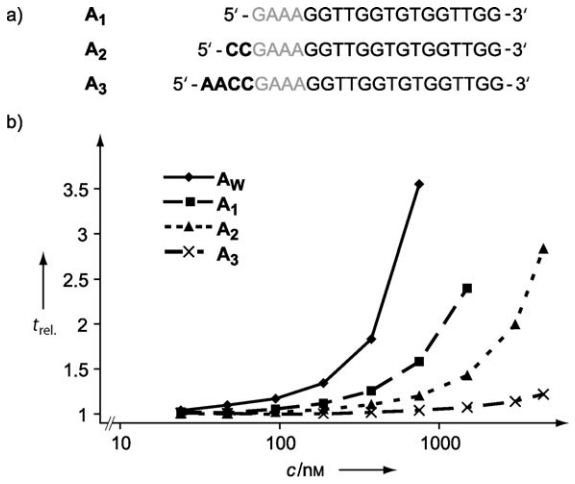

Figure 1. a) The sequence of the 5′-end-elongated aptamers $\mathbf{A_1}$–$\mathbf{A_3}$. b) Results of blood-clotting experiments with the aptamers $\mathbf{A_W}$ and $\mathbf{A_1}$–$\mathbf{A_3}$ (relative blood-clotting time versus aptamer concentration, see the Supporting Information for experimental details).

a GAAA sequence, which builds a stable GNRA tetraloop motif (N = any nucleotide, R = purine). We then performed filter-binding experiments with $\mathbf{A_1}$–$\mathbf{A_3}$ to determine which of the variants are still able to bind to thrombin (Table 1). The

Table 1: Dissociation constants (in nM) of aptamer variants determined by filter-binding experiments.

	Without irradiation	With irradiation[a]
$\mathbf{A_W}$	124 ± 16	n.d.
$\mathbf{A_1}$	155 ± 23	n.d.
$\mathbf{A_2}$	333 ± 44	n.d.
$\mathbf{A_3}$	> 5000	n.d.
$\mathbf{A_4}$	166 ± 12	> 5000
$\mathbf{A_5}$	297 ± 44	> 500

[a] n.d. = not determined.

elongation of the $\mathbf{A_W}$ aptamer with the tetraloop-forming stretch ($\mathbf{A_1}$) results in a slightly decreased affinity. The addition of two further dC nucleotides ($\mathbf{A_2}$) decreases the affinity of the aptamer even further, and almost no affinity was detected after addition of two more dA nucleotides ($\mathbf{A_3}$).[8] Thus, we conclude that already in the aptamer variant $\mathbf{A_3}$, the hairpin rather than the G-quadruplex conformation is predominant.

Furthermore, we analyzed which of the aptamer variants inhibits thrombin-mediated blood clotting in human plasma. As depicted in Figure 1b, aptamer $\mathbf{A_1}$ is still active. However, in comparison with $\mathbf{A_W}$, the same effect is obtained only at higher concentrations. This result has to be kept in mind because even with a completely inactive antisense region the resulting aptamer can only be as effective as $\mathbf{A_1}$. Aptamer $\mathbf{A_2}$ showed only very little remaining activity, and $\mathbf{A_3}$ was found to be inactive within error limits. Thus, the data obtained from the blood-clotting studies are in good accord with the results observed in the interaction studies.

On the basis of these data we decided to extend our toolbox of "caged" deoxynucleotides that can be activated by light to the residue dC^{NPE} (see Scheme 1c).[9] A protected phosphoramidite of dC^{NPE} was used to synthesize the caged aptamer–antidote chimeras $\mathbf{A_4}$ and $\mathbf{A_5}$ (Figure 2a; see the Supporting Information for synthetic details).

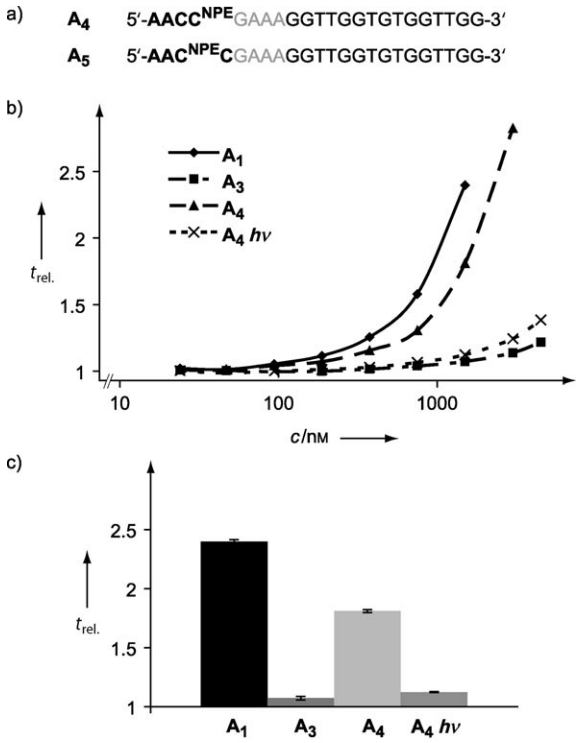

Figure 2. a) The sequence of the caged aptamer–antidote chimeras $\mathbf{A_4}$ and $\mathbf{A_5}$. b) Results of the blood-clotting experiments with the aptamers $\mathbf{A_1}$, $\mathbf{A_3}$, and $\mathbf{A_4}$ (before and after irradiation, relative blood-clotting time versus aptamer concentration, see the Supporting Information for experimental details). c) For a better comparison, the values $t_{rel.}$ in (b) are shown at an aptamer concentration of 1500 nM.

$\mathbf{A_4}$ and $\mathbf{A_5}$ were analyzed for their ability to bind to thrombin and to inhibit thrombin-dependent blood clotting. As demonstrated in Figure 2b and c, $\mathbf{A_4}$ is indeed able to prolong the blood-clotting time in a concentration-dependent manner and with only slightly less efficiency than $\mathbf{A_1}$. As anticipated, $\mathbf{A_4}$ becomes inactive upon irradiation—except at very high concentrations for which some remaining activity could be detected. $\mathbf{A_5}$, however, remained inactive before and after irradiation (data not shown). Circular dichroism (CD) spectra of the aptamers in this study can be found in the Supporting Information. From a photochemical standpoint, it was interesting for us to see how well the dC^{NPE} residue performed in the photodeprotection step. Generally, one might have anticipated from the literature that the removal of the NPE group would create problems because of the often-encountered difficulty to photocleave C–N bonds.[10] However, with our experimental setup (360 nm from three UV LEDs of ca. 100 mW each), 100 pmol of $\mathbf{A_4}$ could be deprotected in 7 s, and the deprotection step was remarkably clean.

For the experiments discussed so far in the text, the irradiation of the caged aptamers was performed before the beginning of the experiment. Thus it was unclear whether a preformed complex of $\mathbf{A_4}$ and thrombin could also be destroyed by irradiation. Figure 3 shows the respective blood-clotting and filter-binding assays, which show that the photodeactivation also functions when $\mathbf{A_4}$ is already bound to thrombin—a fact that will be important for applications in vivo.

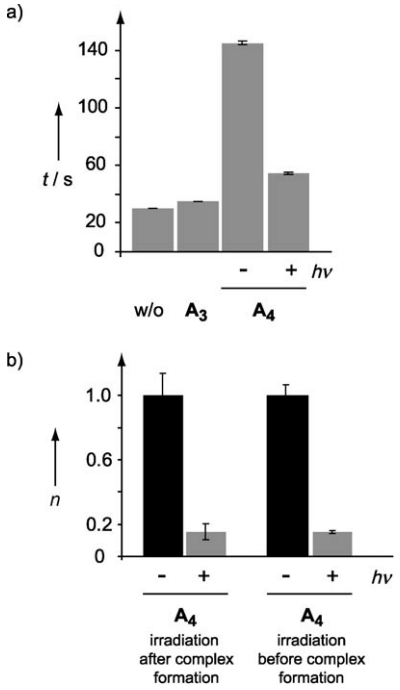

Figure 3. a) Results of blood-clotting assays in which the complex of thrombin and $\mathbf{A_4}$ was irradiated, as well as control experiments with $\mathbf{A_3}$ and without (w/o) aptamer (concentration of aptamer: 3 μM). b) Results of filter-binding assays in which the irradiation was performed before and after formation of the complex with thrombin (*y* axis: relative amount of $\mathbf{A_4}$ bound, concentration of thrombin 150 nM).

In conclusion, after having demonstrated that it is possible to activate an aptamer with light,[5,6] we have now demonstrated that deactivation with light is also possible by incorporating a caged antisense region. Before photodeactivation, the caged chimera showed only about half of the activity of the unmodified wild-type aptamer, but this activity can easily be compensated by increasing the concentration. It is more important that the aptamer becomes virtually inactive upon irradiation. As before,[6] the location of the caged residue (in this case the caged dC^{NPE}) is very important. The new aptamer described herein combines the advantages of a highly specific anticoagulant with rapid and effective control of its function. These characteristics are of potential benefit to patients who are at high risk of anticoagulant-associated bleeding and in clinical situations in which rapid reversal of the anticoagulant functions is required, such as in anticoagulation of extracorporeal circulation devices. Furthermore, this approach will be of general applicability to control the activity of nucleic acid based modulators of protein function in a spatiotemporal fashion.

Received: June 12, 2006
Published online: September 19, 2006

Keywords: antisense agents · aptamers · blood clotting · caged compounds · DNA

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[9] Literature on caged compounds in general and caged oligonucleotides in particular can be found in our recent review article: G. Mayer, A. Heckel, *Angew. Chem.* **2006**, *118*, 5020–5042; *Angew. Chem. Int. Ed.* **2006**, *45*, 4900–4921.
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